ASSESSMENT OF HISTO-CYTOPATHOLOGICAL RESPONSES IN THE GILLS AND DIGESTIVE DIVERTICULAE OF COMMERCIAL BIVALVES ALONG THE KERALA COAST AS INDICATORS OF ENVIRONMENTAL QUALITY.

FINAL SYNOPSIS

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FINAL SYNOPSIS

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CHAPTER 1

GENERAL INTRODUCTION

Bivalves are one of the most diverse and abundant group of molluscs with two-halved shells, composed mostly of calcium carbonate, connected by a flexible ligament. They are filter-feeders, sedentary and sentinel species with resistance to dynamic environmental conditions. They are also able to accumulate toxic substances from the environment. These attributes make bivalves ideal candidates for biomonitoring programs (Helmholz et al., 2016). India has a very long coastline spreading over 7500 Km between the Arabian Sea in the west and the Bay of Bengal in the east. Clams, mussels, and oysters are the three most important commercially exploited bivalves in India. A considerable proportion of India's bivalve production is from the state of Kerala; with Vembanad and Ashtamudi backwaters being the main harvesting areas, generating more than 80,000 tonnes of bivalves in 2018 (Chinnadurai et al., 2021). These species are popular among locals and tourists as delicacies that are nutrient and protein-rich, as well as a source of income for a large number of communities that rely on fisheries. Bivalves are often used as bioindicators of aquatic toxins for tracking toxicity and risks in coastal habitats because they easily reflect the abiotic or biotic status of an ecosystem. Aquatic pollution is one of the most serious issues confronting the world today, and it is increasing with each passing year, causing devastating and irreversible damage to the habitat and associated life. In recent years, the Kerala coast has also been under the threat of pollution due to the discharge of industrial effluents, agricultural runoffs, domestic sewage, marine litter, tourism, and urbanization (Sitaram, 2018). Over the last two decades, bivalve biomarker research has advanced significantly and is now widely used in screening aquatic pollution. Several studies have used biological markers as reliable and effective early warning indicators of ecosystem depletion (Cuevas et al., 2015; Costa et al., 2013). Initial reports on bivalves as an indicator of aquatic pollution began to appear in the 1960s and 1970s (Seymour, 1966; Shimizu et al., 1971). One of the earliest environmental monitoring initiatives that used living organisms over a wide geographic area was the infamous mussel watch, which started in the United States of America in 1976. It involved screening of radionuclides, halogenated hydrocarbons, petroleum hydrocarbons, and heavy metals in four marine bivalves (*Mytilus edulis*, *Mytilus californicus*, *Crassostrea virginica*, and *Ostrea equestris*) at more than 100 locations along the coast of North America (Stankovic et al., 2012). The most widely used bivalve species for biomonitoring include mussels (*M. edulis*, *Mytilus galloprovincialis*, and *M. californicus*), oysters (*Ostrea edulis* and *C. virginica*), and clams (*Macoma balthica*, *Mercenaria mercenaria*, and *Venerupis* sp) (Oehlmann & Schulte-Oehlmann, 2003). The term ‘biomarker’ refers to any physiological, cellular, molecular, biochemical, or morphological indicators that provide insights into the interaction between a specific organism and its surrounding environment (Gagné et al., 2004). They will indicate whether an organism has been exposed to pollutants and how much of a reaction the organism has had. These biomarkers can thus be used in a predictive manner, allowing the implementation of bioremediation solutions before irreversible harm and ecological consequences arise (Cajaraville et al., 2000). Multiple biomarker research has been extensively conducted in various regions worldwide, but its exploration remains

relatively limited in India (Hariharan et al., 2016; Arockia Vasanthi et al., 2012). As a result, the present research was carried out to investigate the current status and implications of deteriorating environmental quality along the coast of Kerala by using three distinct commercially and economically significant bivalve species viz., edible oyster (*Magallana bilineata*), green mussel (*Perna viridis*) and the black clam (*Villorita cyprinoides*) as bioindicators.

Objectives of the study

- To quantitatively evaluate the histological lesions and alterations in bivalves as markers of environmental quality.
- To evaluate the accumulation of heavy metals in bivalves.
- To isolate, enumerate, and identify the nature of microplastics in surface water and soft tissues of bivalves.
- To investigate the specificity of discrete cytopathological responses in bivalve species as a biomarker of environmental quality.
- To evaluate the *in vivo* genotoxic reactions in bivalves and to explore the ability to be used to monitor pollution and environmental quality.

Study area and sample collection

The bivalve samples for the investigation were collected from five distinct sites along the south-west coast of India: Ashtamudi Lake in Dalavapuram (S1), Kayamkulam Lake (S2), Vembanad Lake in Muhamma (S3), Periyar River in Aluva (S4), and Vembanad Lake in Sathar Island (S5) (Fig. 1.4). The bivalve species collected from different sites represented the primary bivalve fishery in those regions. These sites were chosen for sampling due to their ecological and economic significance. Further, sites S1, S3, and S5 are listed as wetlands of international importance according to the Ramsar Convention for the conservation and sustainable use of wetlands (Convention on wetland, 2024).

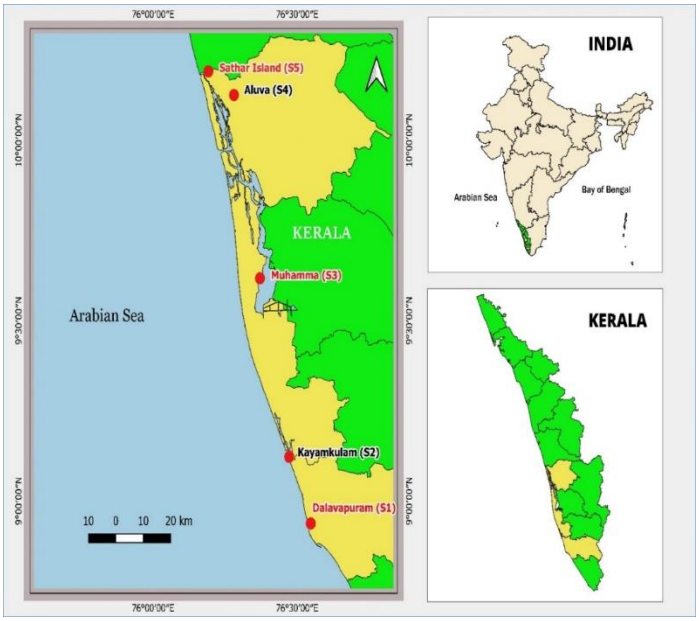

Fig. 1.4. Map showing sampling locations (red fonts indicate Ramsar sites)

Bivalve samples of *M. bilineata, P. viridis,* and *V. cyprinoides* were collected and carried alive to the laboratory in water-soaked cotton. A total of 756 samples were examined and the sampling details are shown in Table 1.1.

Aquatic realms	Site	GPS coordinates	Bivalve species sampled	Number of samples collected							
				2019			2021			2022	
				Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon
Ashtamudi Lake	Dalavapuram (S1)	8°56'15.9"N; 76°32'51.0"E	*M. bilineata*	26	20	20	15	15	15	9	6
Kayamkulam Lake	Kayamkulam (S2)	9°07'21.7"N; 76°28'21.6"E	*M. bilineata*	26	20	20	15	15	15	9	6
			P. viridis	26	20	20	15	15	15	9	6
Vembanad Lake (Freshwater zone)	Muhamma (S3)	9°37'03.8"N; 76°22'16.7"E	*V. cyprinoides*	26	20	20	15	15	15	9	6
Periyar River	Aluva (S4)	10°07'33.1"N; 76°16'51.7"E	*V. cyprinoides*	26	20	20	15	15	15	9	6
Vembanad Lake (Saltwater zone)	Sathar Island (S5)	10°11'29.3"N; 76°11'32.4"E	*P. viridis*	26	20	20	15	15	15	9	6

Table1.1 Details of sampling sites and number of samples collected seasonally.

Identification of bivalves

The bivalves were identified using both genetic information and morphological traits such as shell color, number of adductor muscles, hinge size, umbo, and tooth position (Narasimham, 2005). For molecular characterization, DNA was extracted from the mantle tissue using a tissue DNA extraction kit (Qiagen). To barcode the DNA, the cytochrome C oxidase subunit (CO1) gene was amplified employing a Vereti thermal cycler (Applied system USA) using LCO1 and HCO1 primers (Folmer et al.,1994). The PCR conditions consisted of an initial denaturation step at 94^0C for 3 min, followed by 35 cycles of denaturation at 94^0C for 30 sec, annealing at 46^0C for 60 sec, extension at 72^0C for 30 sec, and a final extension at 72^0C for 5 min. The PCR products were then purified and Sanger sequenced (Agrigenome, India). The resulting sequences were aligned using DNA star and BLAST was used to find and compare matching sequences in the NCBI database. The BOLD identification engine was used to validate the species.

CHAPTER 2

EVALUATION OF GENERAL HISTOPATHOLOGICAL RESPONSES AND HEAVY METAL ACCUMULATION.

2.1 Summary

To evaluate environmental quality, a semi-quantitative histopathological index was adopted to examine the histological changes in the digestive gland and gills of three bivalve

species: edible oyster (*M. bilineata*), green mussel (*P. viridis*), and black clam (*V. cyprinoides*). Tubular vacuolation, fibroma, haemocytic infiltration, necrosis, lipofuschin aggregates, and lamellar disorganization, were the notable tissue alterations along with the presence of prokaryotic inclusions, and protozoan parasites. Additionally, the digestive glands of bivalves were examined for the presence of ten different trace metals such as cadmium (Cd), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb), zinc (Zn), iron (Fe), mercury (Hg), cobalt (Co), and chromium (Cr). Seasonal variations were noted in all reaction patterns in the digestive gland throughout the sampling sites, with post-monsoon showing the highest indices. Bivalves from Muhamma, Vembanad Lake (S3), and Aluva, Periyar River (S4) had significantly higher indices for all the reaction patterns in the digestive gland. Dalavapuram, Ashtamudi Lake (S1), and the Aluva, Periyar River (S4) exhibited the highest indices for cellular changes and parasitosis in gills among the sampled sites. Also, Muhamma, Vembanad Lake (S3), and Aluva, Periyar River (S4) had the most elevated overall histopathological indices for the digestive gland and gills. The highest metal pollution index was found in *V. cyprinoides* from Muhamma, Vembanad Lake (S3), followed by *M. bilineata* from Dalavapuram, Ashtamudi Lake (S1) and Kayamkulam Lake (S2). Principal component analysis also indicated that Muhamma, Vembanad Lake (S3) stood out with the highest metal pollution index. A positive relation was found between histological changes in the digestive gland, heavy metals, and the season and sampling sites. The study revealed that the histopathology index values indicated mild to moderate contamination at all examined sites.

2.2 Introduction

Bivalves have long been used to evaluate aquatic toxicity, particularly caused by heavy metals. Monitoring pollution by examining bivalve tissues has proven to be more effective than analyzing water and sediment samples (Phillips and Segar, 1986). The presence of heavy metals in bivalves provides a time-integrated measure of contaminants bioavailability (Phillips and Segar, 1986). The concentration of essential and nonessential trace metals that bivalves can accumulate in their tissues can be up to 0.1 million times higher than those in their environment (Farrington et al., 2016). This is primarily because of their limited ability to metabolize these metals efficiently (Luoma and Rainbow, 2005). The health risks associated with high exposure to trace elements through seafood consumption are particularly significant in coastal regions. Heavy metal toxicity in seafood is a major global issue, with particularly severe implications in developing countries (Taweel et al., 2013). The digestive gland and gill tissues of bivalves are vital in the accumulation and detoxification of heavy metals. Bivalve gills play crucial roles in respiration and feeding. Additionally, as the primary interface between the organism and its surroundings for the uptake of pollutants, they are a sensitive organ for tracking contaminants (Rainbow, 2002). The digestive gland, also called the hepatopancreas is an integral organ for digestion and absorption. It produces digestive enzymes that break down food particles, which later get absorbed by its epithelial cells (Rainbow, 2002). Besides endo-cellular digestion, the digestive glands of bivalves also accumulate and detoxify various chemicals from the aquatic environment. Many field studies have demonstrated that metal concentrations in bivalve tissues can be effectively utilized as

indicators for monitoring heavy metal pollution in aquatic environments (Wagner & Boman, 2004; Krismonita et al., 2023). Histopathological markers are basic and indicate individual health, which can be generalized to the population (Au, 2004). Several investigations have established the effect of histo-morphological changes in the gills and digestive gland to pollutants (Arockia Vasanthi et al., 2012). In a few regionally relevant bivalve species, a semi-quantitative technique for measuring histological alterations has already been reported (Costa et al., 2013; Cuevas et al., 2015). There have been a few biomonitoring studies done in the Vembanad and Ashtamudi wetland system on the southwest coast of India, wherein they have only looked at trace metals in the bivalve soft tissues (ShibiniMol, Raveendran and Sujatha, 2015; Ragi et al., 2017). Apart from that no research has been undertaken to assess environmental quality using histopathological indices as pollution markers in bivalves. Hence, the current study aims 1) to measure the health of ecosystems using a semi-quantitative approach for determining histological abnormalities in bivalve's digestive glands and gills; 2) to understand the geographical variance in histopathological indices in bivalves from various sampling zones; 3) to determine whether there is a correlation between histopathological indices of the digestive gland and the presence of trace metals in bivalves sampled.

2.3 Materials and Methods

2.3.1 Histopathological evaluation

A total of 360 bivalve samples were analysed for histopathology. Samplings were made during pre-monsoon, monsoon and post-monsoon seasons of the year 2019 The gill and digestive gland from each individual were dissected and kept in 10% neutral buffered formalin for 48h (Shaw and Battle, 1957). After that, the tissues were rinsed in running tap water overnight, processed in a series of sequentially stronger alcohols, cleared in xylene, and finally embedded in paraffin wax. Sections of 3-5 µm thickness were cut using a semiautomatic microtome (Leica Wetzlar, Germany) and stained with Harris hematoxylin and acidic eosin for conventional histopathological examination (Howard and Smith, 1983). The stained sections were observed under a Nikon Eclipse 80i microscope and conspicuous morphological features were captured.

2.3.2 Evaluation of histopathological condition indices

To evaluate the histopathological condition indices (HPI), an approach of scoring histopathological changes in fish proposed by Bernet et al. (1999) and then revised by Costa et al. (2013) was adopted in this work. Briefly, each morphological variation was assigned a weightage ranging from 1 (lowest severity) to 3 (highest severity) depending on its biological importance (Table 2.1.). Following that, the degree of spread of each weighted lesion was assigned a score ranging from 1 to 6, where 1 indicated minimal changes and 6 indicated extensive changes. The severity of morphological aberrations was determined using the equation:

$$I_h = \frac{\sum_1^J W_J a_{jh}}{\sum_1^j M_J}$$

where Ih is the HPI for the individual species h; Wj the weight of the j^{th} histopathological alteration; ajh is the score value for the extent of dissemination of the j^{th} alteration in h^{th} individual, and Mj is the attributable maximum (weight multiplied with maximum score) for the j^{th} alteration. HPI was computed independently for each bivalve species' gills and digestive gland. A total of twenty abnormalities in the digestive glands and gills were assessed. Tubular changes, intertubular changes, and parasitosis were the three categories used to describe the reaction pattern in the digestive gland. For the gills, the alterations were categorized as parasitosis and cellular changes. Weightage(W) of the recorded pathologies in the digestive glands and gills were presented in table 2.1. Mean HPI per reaction pattern was compared across sampling seasons and sites. The change in the HPI of the digestive gland and gills with and without parasites was also assessed across sites and seasons. When the HPI was within 0.00 to 0.30, 0.31 to 0.60, and 0.61 to 1.0, it was classified as low, moderate, and high, respectively. For each sampling, the prevalence of different lesions was computed as (total number of affected individuals / total number of individuals studied) x 100. The overall mean HPI for bivalve digestive glands and gills was also calculated. The mean HPI of oysters and mussels from Kayamkulam Lake (S2) was taken into consideration for comparison when evaluating the overall HPI and regional variance in HPI with and without parasites.

	Reaction pattern	Alterations	W
Digestive gland	Tubular changes	Hemocytic infiltration	1
		Hemocytic aggregates	1
		Lipofuschin aggregates	1
		Tubular atrophy	3
		Necrosis	3
		Vacuolation	1
	Intertubular changes	Hemocytic infiltration	1
		Hemocytic aggregates	1
		Lipofuschin aggregates	1
		Fibroma	2
	Parasitosis	Bacteria	2
		Protozoans	3
Gill	Cellular changes	Vacuolation	2
		Hemocytic infiltration	3
		Granulocytoma	1
		Lipofuschin aggregates	1
		Fibroma	2
		Lamellar deformation	1
	Parasitosis	Bacteria	2
		Protozoans	3

Table

2.1. Weightage(W) of the recorded pathologies in the digestive glands and gills.

2.3.3 Evaluation of heavy metals in the digestive gland of bivalves

The digestive gland of bivalves was analysed for the presence of heavy metals such as Cd, Cu, Mn, Ni, Pb, Zn, Fe, Hg, Co, and Cr in order to understand the correlation between the presence of heavy metals and tissue alteration. An atomic absorption spectrometer (AAS) facility installed at the ICAR-Central Institute of Fisheries Technology, Kochi, India was used for heavy metal evaluation. To assess how different sites vary in the value of trace metals found in bivalves, the concentration of various heavy metals in the digestive diverticula of bivalves was converted into a single value as the metal pollution index (MPI) using the equation formulated by Usero, (1997) to assess differences in the distribution of trace metals in bivalves across different sites:

$$MPI = (C_{Cr}\ X\ C_{Cu}\ X\ C_{\ Mn}\ XC_{\ Ni}\ X\ C_{\ Pb} X\ C_{Zn} X\ C_{\ Fe} X\ C_{Cr}\ X\ C_{hg}\ XC_{co})^{\ 1/10}$$

2.3.4 Statistical analysis

The data analysis was done using SPSS 13.0 for Windows. The normality and homogeneity of the obtained results were assessed through Kolmogorov-Smirnov and Shapiro-Wilk tests. To examine significant differences in histopathological indices between seasons and zones, non-parametric tests such as Kruskal-Wallis, and Wilcoxon matched pair tests were employed. Principal component analysis (PCA), a multivariate statistical technique used to reduce a set of interrelated variables with high dimensionality into a lower dimension, was applied to assess the correlation between the concentrations of different heavy metals in the digestive gland and various histomorphometric parameters during different seasons. The *factoextra R* package was used for this analysis (Kassambara and Mundt, 2020).

2.4 Results and Discussion

2.4.1 Histopathology of digestive gland

The predominant histological alterations observed in the digestive gland of bivalves from all the investigated sites included vacuolation in the tubular epithelium, haemocytic infiltration, lipofuscin aggregates, and parasitosis. A higher incidence of severe tubular vacuolation (Fig.2.1a) was seen in mussels and clams (70-100%). Increased vacuolation of the digestive gland tubule was documented in *P. viridis* exposed to coastal pollutants (Khan et al., 2015) and in *M. edulis* subjected to trace metal contamination (Wedderburn et al., 2000). Clams collected from Muhamma, Vembanad Lake (S3) showed a prevalence value of 80-90% for haemocytic infiltration (Fig2.1a). Lipofuscin aggregates were found in the digestive tubule epithelium and intertubular tissue as yellowish granular pigments (Fig. 2.1b) (5-100%). Lipofuscin, a byproduct of lipid peroxidation resulting from the damage of polyunsaturated fatty acids by reactive oxygen species (Wolke et al., 1985), was also reported in *P. viridis* exposed to coastal pollutants (Khan et al., 2015). Atrophy, narrowing of the epithelial cell layer, and enlargement of the tubular lumen (Fig. 2.2c) were frequently observed in clams from Muhamma, Vembanad Lake (S3), and oysters from Kayamkulam Lake (S2). the digestive tubules of *M. bilineata* from sites with metal pollution also

demonstrated tubular atrophy (Gold-Bouchet et al., 1995). Ros et al. (1995) correlated digestive gland tubular atrophy to the first signs of tubular necrosis in mussels exposed to cadmium. Necrosis and intertubular fibroma (Fig. 2.1d) were also observed in the sampled bivalves. The most parasitized organisms were clams from Muhamma, Vembanad Lake (S3) and Aluva, Periyar River (S4). Nematopsis-like parasites were found in clams from Muhamma, Vembanad Lake (S3), and Aluva, Periyar River (S4) with a prevalence of 20-100% (Fig. 2.1e). These parasites were observed in all of the mussels collected from Sathar Island, Vembanad Lake (S5) during post-monsoon in the tubular lumen and intertubular connective tissue of the digestive diverticulae as numerous oval oocytes (Fig. 2.1f). They infect various bivalve species globally without causing obvious harm to their hosts(Bower et al., 1994). Bivalves are secondary hosts for these parasites, whereas marine arthropods act as final hosts (Lauckner, 1983). Intracellular prokaryotic inclusions were also common across the study zone, however the prevalence (40-70%) was higher in oysters collected from Dalavapuram, Ashtamudi Lake (S1) and Kayamkulam Lake (S2) (Fig. 2.2.a, b). According to Vázquez & Cremonte (2017) prokaryotic inclusions are usually related to rickettsia or chlamydia-like organisms, which are typically pleomorphic having coccoid to ovoid structure. They are typically characterized as infections with low pathogenicity that elicit minimal immune responses (Carballal et al., 2001).

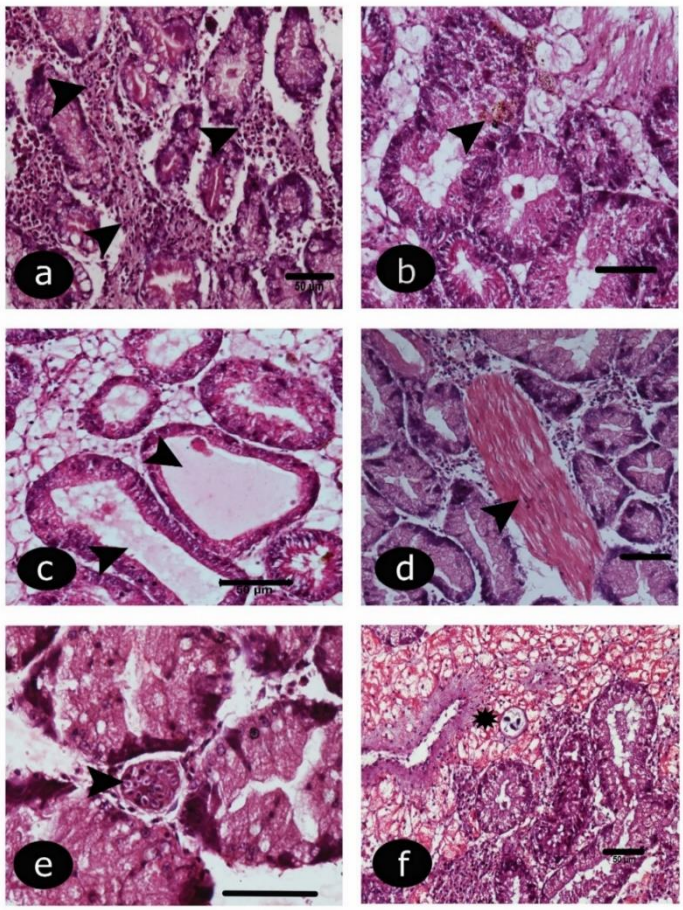

Fig. 2.1. Microphotographs of histological anomalies in the digestive gland of bivalves collected from different sites: (a) haemocytic infiltration and tubular vacuolation with disorganized epithelial lining (arrow head); (b) lipofuscin aggregates in the tubular and intertubular tissues (arrow head); (c) tubular atrophy (arrowhead); (d) intertubular fibroma (arrow head); (e) Nematopsis like parasites in the intertubular connective tissue (arrow head); (f) Nematopsis oocytes (asterisk).

2.4.2 Histopathology of gills

Pathological changes observed in bivalve gills included epithelial cell vacuolization, mucous secretory cell degeneration, and hemocytic infiltration with lamellar disorganization. The clams from Muhamma, Vembanad Lake (S3) had the highest prevalence of gill alterations, while oysters and mussels from Kayamkulam Lake (S2) had the lowest prevalence. The observed changes included fusion of secondary lamellae, infiltration of hemocytes, and a reduction in the space between water tubes and the inter-lamellar junction (Fig.2.2c). The investigation on American oysters (*Crassostrea virginica*) from locations affected by petroleum hydrocarbons and heavy metals indicated damage to their gills, such as oedema and epithelial hyperplasia. These alterations were strongly correlated to the presence of unresolved hydrocarbon fraction (UCM) and high levels of cadmium in the water(Gold-bouchot et al., 1995). Nematopsis-like parasites were observed frequently in clams from Muhamma, Vembanad Lake (S3), and Aluva, Periyar River (S4), as well as mussels from Sathar island, Vembanad Lake (S5) during the post-monsoon season (Fig. 2.2d). Gills were the heavily parasite-infested organ analysed, similar to the findings of Soto et al. (1996) in 12 bivalves from Ria de Vigo, Spain. A single clam from Aluva, Periyar River (S4) exhibited haemocytic nodules during the post-monsoon season (Fig. 2.2e). Fibromas were found in 50-80% of clams collected from Muhamma, Vembanad Lake (S3). One mussel sampled from Kayamkulam Lake (S2) showed disseminated neoplasia with large haemocyte aggregates and pleomorphic nuclei (Fig. 2.2f). This disease was previously detected in 15 marine bivalve molluscs worldwide (Galimany & Sunila, 2008).

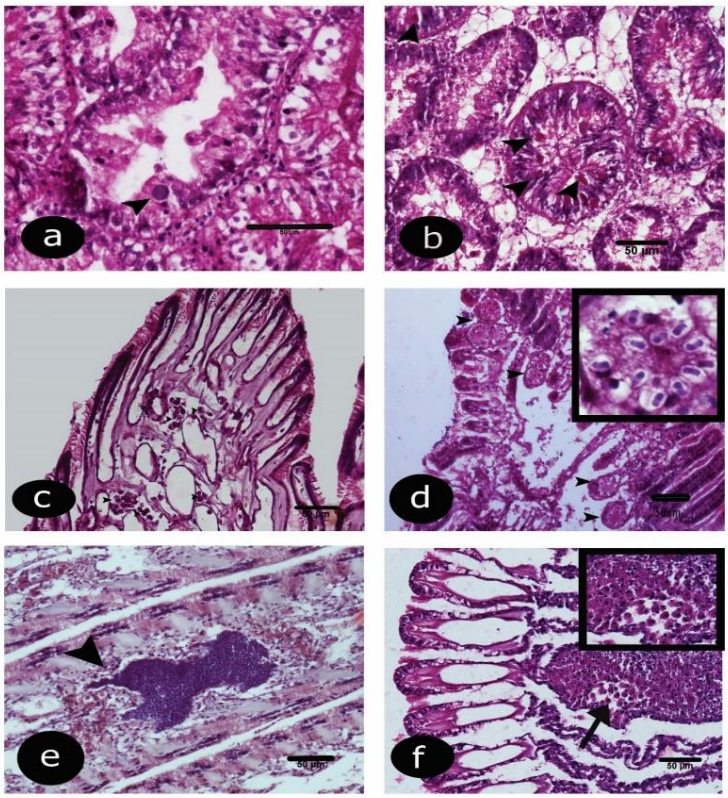

Fig. 2.2. Microphotographs of **h**istological anomalies in the digestive gland and gills of bivalves collected from various sites: (a, b) prokaryotic inclusions in the tubular epithelial cells (arrowhead); (c) lamellar fusion with epithelial cell hypertrophy in the gills (arrowhead); (d) Nematopsis-like parasites in the epithelial cells of the gills (inset: oocysts) ; (e) haemocytic nodule in the gill lamellae (arrowhead); (d) disseminated neoplasia (arrow) in the gills (inset: aggregates of large hemocytes with a prominent nucleus).

2.4.3 Evaluation of histopathological condition indices

The degree of histological alterations varied from low to moderate. Every reaction pattern displayed seasonal fluctuations between sites. Nevertheless, the reaction pattern with the highest HPI was tubular alterations. The elevated tubular HPI was recorded in all sites during the post-monsoon, except Dalavapuram, Ashtamudi Lake (S1), which displayed significantly low values ($P < 0.05$). In terms of intertubular changes, post-monsoon mussels, and clams had significantly ($P < 0.05$) higher HPI than pre-monsoon and monsoon, whereas the HPI in clams sampled from Muhamma, Vembanad Lake (S3) remained consistent throughout three seasons. In the case of parasitosis, clams from Aluva, Periyar River (S4) had regular infections with no seasonal fluctuations. In all the sampling sites and seasons, histopathological indices in the gills exhibited lower values than in the digestive gland. Consistent parasite infection was noticed in the gills of Muhamma, Vembanad Lake (S3), and Aluva, Periyar River (S4) clams, similar to the digestive gland. Bivalves from Sathar island, Vembanad Lake (S5), and Aluva, Periyar River (S4) showed a significant ($P < 0.05$) difference in cellular HPI during post-monsoon. Besides, there was no significant seasonal variation in the cellular HPI of the gills across sites. The average digestive gland and gill HPI per reaction pattern across various sites of sampling is presented in (Fig.2.3). The oysters from Dalavapuram, Ashtamudi Lake (S1) exhibited the highest cellular HPI, which was significantly ($P < 0.05$) distinct from other sites, except for the mussels from Sathar Island, Vembanad Lake (S5). The highest incidence of parasitic infection was observed in clams from Aluva, Periyar River (S4). The histopathological indices of the digestive gland and gills exhibited seasonal and regional variations. The clams collected from sites Aluva, Periyar River (Z4) and Muhamma, Vembanad Lake had extremely high HPI levels compared to bivalves from other sites. This could be attributed to the substantial influx of untreated hazardous effluents from nearest agricultural, household, and industrial operations(Sobha et al., 2011). To figure out the influence of parasites on histo-morphological changes, the HPI is to be measured with and without parasites (Cuevas et al., 2015).The calculation of HPI with and without parasites indicated a similar seasonal pattern for the digestive gland and gills. In contrast, the HPI of bivalves without parasites exhibited an increasing pattern across sites, except gill HPI of clams from Aluva, Periyar River (S4), which had the lowest levels during post-monsoon. Therefore, it seems that the real histological alterations were inflated by parasites in moderately contaminated zones. The overall mean digestive gland and gill HPI for each site is presented in Fig.2.4.

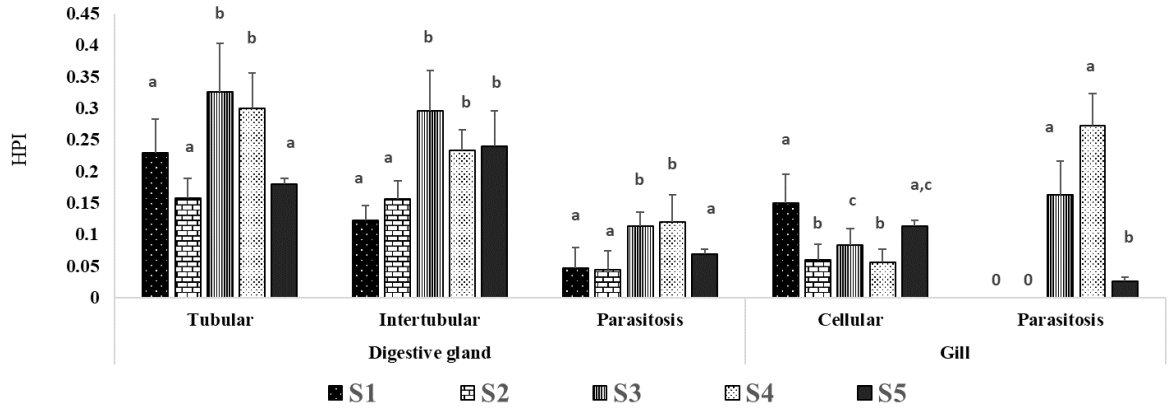

Fig. 2.3. Histopathological condition indices of the digestive gland and gills per reaction pattern across sampling sites (bars with different letters have significant differences within a sampling site, vertical bars indicate standard error).

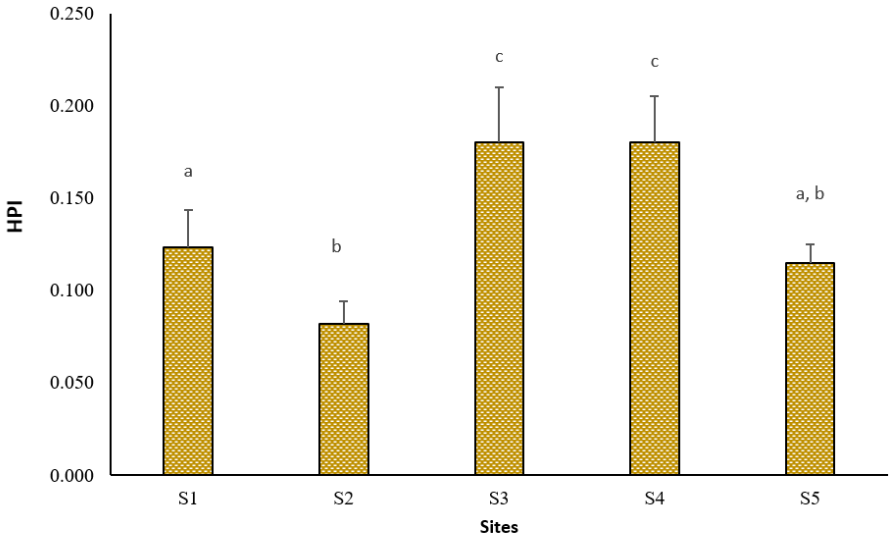

Fig.2.4. Mean overall histopathological condition indices (±95% confidence interval) for different sites (bars with different letters have significant difference between sites, vertical bars indicate standard error).

2.4.4. Evaluation of heavy metals

The order in which metals were found was Fe>Zn>Mn>Cu>Cd>Ni>Pb>Cr. Among the metals tested, Hg and Co appeared to be below the detection limit in all sites. Fig.2.5. illustrates the metal pollution index of bivalves from each examined site across different seasons. Clams from Muhamma, Vembanad Lake (S3) had the greatest MPI values during post-monsoon, followed by oysters from Dalavapuram, Ashtamudi Lake (S1) and Kayamkulam Lake (S2) during monsoon. The present study analysed six essential metals (zinc, copper, iron, chromium, manganese, and cobalt) that are necessary for various biochemical and physiological activities of animals. The other metals (nickel, mercury, cadmium, and lead) are non- essential. They have the potential to become harmful even at very low concentrations (Kamaruzzaman et al., 2011). The histopathological indices from Muhamma, Vembanad Lake (S3) and Aluva, Periyar River (S4) reflect the contamination patterns of these ecosystems, both of which are considerably contaminated with Fe and Mn. Conversely, Kayamkulam Lake (S2) exhibited a significantly lower HPI than S3 and S4. S2, which is the Ayiramthengu part of the Kayamkulam Lake near the harbor is known to be pristine(Krishnan & Jaya, 2014).

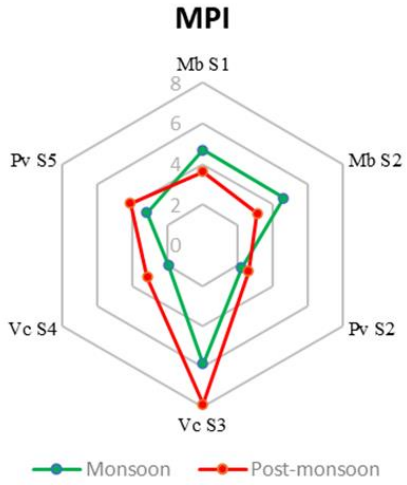

Fig.2.5. Metal pollution index of the digestive gland of bivalves from various sites during different seasons (Mb: *M. bilineata*, Pv: *P. viridis*, Vc: *V. cyprinoides)*.

2.4.5. Principal component analysis

PCA was employed for all digestive gland histopathological reaction patterns and individual heavy metal data (11 variables) to obtain a comprehensive picture of the metal contamination profile and histological changes of bivalves at the five sampling sites. The first principal component (PC1, denoted by Dim1) is a linear combination of original variables that reflects the most variance in the data set. The second principal component (PC2, denoted by Dim2) is a linear combination of original variables, showing the second direction of more significant difference and is unrelated to PC1. PC1 accounted for 45.5% of the total variability in the data and included heavy metals such as copper, manganese, nickel, chromium, and iron, as well as digestive gland reaction patterns like parasitosis, tubular

changes, and intertubular changes. On the other hand, PC2 contributed 18.4% of the variability and consisted of heavy metals such as zinc, cadmium, and lead. PC1, there was also a significant positive relationship between heavy metals and HPI. Trace metals are recognised as the primary sources of contamination in marine and estuarine environments(Salánki et al., 2003). The classification of HPI by site showed that there were more significant changes in the tubular and intertubular tissues of the digestive gland of clams from Muhamma, Vembanad Lake (S3) and Aluva, Periyar River (S4) accompanied by a higher occurrence of parasitosis, and presence of trace metals compared to other sites. The Periyar river flows into the Cochin backwater system, which is the largest estuarine system in South Asia and plays a crucial role in fisheries and tourism. The chemical, metal processing, and petrochemical industries located along the Aluva Periyar riverbanks (S4) discharge significant amounts of effluents(Priju & Narayana, 2007; Salas et al., 2017). Previously, this site was also reported to have higher values of copper, cadmium, and zinc in the sediment, along with a higher MPI (Priju & Narayana, 2007). In Dalavapuram, Ashtamudi Lake (S1), elevated levels of iron and zinc in the surface waters have been previously documented. The high iron concentrations are attributed to the mixing of untreated domestic and industrial waste (Karim & Sherly Williams, 2015). Overall, the higher HPI values in this study are likely to be associated with the elevated levels of heavy metals found in the sampling sites.

CHAPTER 3

EXTRACTION, QUANTIFICATION, AND IDENTIFICATION OF MICROPLASTICS FROM BIVALVES.

3.1 Summary

The escalating generation of plastic waste and its ensuing accumulation as microplastics in the environment has become a worldwide concern, particularly in coastal ecosystems. The present study attempted to investigate the presence of microplastics in the water and soft tissues of three commercially important bivalves, viz. edible oyster (*Magallana bilineata)*, green mussel *(Perna viridis)*, and black clam *(Villorita cyprinoides)* from five different locations along the southwest coast of India. The highest concentration of microplastics in water was found in samples from the Aluva, Periyar River (163.67 items L^{-1}). Among the bivalves, clams from Aluva, Periyar River (S4) exhibited the highest abundance of microplastics (digestive gland: 22.8g^{-1}; gill: 29.6g^{-1}), While mussels from Sathar Island, Vembanad Lake (S5) had the lowest (digestive gland: 5.6g^{-1}; gill: 8.5g^{-1}). Fibers were the most abundant microplastics detected in the tissue of bivalves analyzed across all the sampling sites. Microplastics smaller than 2 mm were the most prevalent size. Raman spectroscopy confirmed the presence of microplastics such as polypropylene and high-density polyethylene. No correlation was found between shell length, tissue weight, and microplastic abundance. A significant positive correlation was found between the presence of microplastics in water and their accumulation in bivalve tissues. The study provided valuable insights into the effectiveness of using sedentary bivalves to assess aquatic pollution.

3.2. Introduction

Plastic is persistent in the environment and difficult to eliminate, and it is currently one of the most prevalent types of debris observed in the aquatic ecosystems (Sruthy & Ramasamy, 2017). In 2018-19, India produced 3.3 million metric tons of plastic waste, ranking it as the 12th largest contributor to marine litter. This is primarily due to the runoff of plastic waste from garbage dumps into the interconnected system of rivers, lakes, and estuaries (Nikki et al., 2021). Plastic particles smaller than 5mm are categorized as microplastics, and found in the aquatic environment in various shapes and sizes (Thompson et al., 2004). Due to their small size, aquatic organisms frequently mistake microplastics for food. Thus, floating and deposited microplastic particles may be consumed by both deposit and suspension feeders. Microplastics have been identified in various aquatic organisms across different trophic levels, including plankton, crustaceans, bivalves, and fish (Nikki et al., 2021; Cole et al., 2019; Sun et al., 2018). In bivalves ingestion of microplastics causes inflammation(Browne et al., 2008), reduction of energy reserves(Wright et al., 2013), impaired larval growth (Sussarellu et al., 2016), and decreased feeding rates (Xu et al., 2017). While India has extensive study reports on microplastic accumulation in the marine environment, only a few studies have been documented in lake and river ecosystems (Amrutha & Warrier, 2020; Gopinath et al., 2020). Likewise, reports on microplastic contamination in Indian bivalves are scarce (Naidu, 2019; Dowarah et al., 2020). So far, no studies have reported the abundance of microplastics in commercially harvested bivalve species from ecologically significant areas such as the Vembanad and Ashtamudi estuaries along the southwest coast of India. In this context, the present study attempted 1) to evaluate the microplastic contamination in the gills and digestive gland tissues of bivalves; 2) to classify microplastics from various bivalve species based on type, size, and colour; 3) to investigate the relationship between microplastic content in bivalve tissue and water; 4) to compare the morphometric characteristics of bivalves and the abundance of microplastics in tissues.

3.3. Materials and Methods

3.3.1. Microplastic quantification and characterization

A total of 36 bivalves were collected from five different locations and transported alive in an ice box to the laboratory, where the bivalves were kept at -80^{0}C till further analysis. Further, 6L of surface water was also taken from the study zones and stored at 4^{0}C until analysis. Bivalve sampling was carried out in pre-monsoon 2019.

3.3.1.1. Quality control measures

To prevent external contamination, quality control measures were implemented during the microplastic extraction technique (Foekema et al., 2013).

3.3.1.2. Surface water

The wet peroxidation method, as described by Masura et al. (2015) was employed to extract microplastics from water samples. The procedure involved collecting surface water samples in a stainless-steel bucket, filtering them through 0.3mm mesh stainless steel sieves, and transferring the solids to a glass beaker. The solids were then dried at 90^0C for 24 hours and treated with 30% hydrogen peroxide to dissolve the natural compounds present. A saltwater density separation technique using 5M sodium chloride was utilized to separate microplastic through floatation. The water filtered through a 0.3mm mesh size was further filtered using glass microfiber filters with a pore size of 1.2 µm to quantify microplastics smaller than 300 µm. After that, the filters containing microplastics were transferred to glass petri plates lined with aluminium foil and left to dry overnight in an oven at 60^0C. The microplastics were then sorted and analyzed under a stereomicroscope (Nikon SMZ 1000).

3.3.1.3. Bivalve tissues

The enzymatic digestion method was used to extract microplastics from bivalve tissues, following the procedure described by Lusher et al. (2017). In this method, the digestive gland and gill tissues from the sampled bivalves in triplicate (0.3g each) were homogenized in a buffer solution and incubated at 50^0C for 15 minutes. After incubation with Proteinase-K (8mg) for 2 hrs at 50^0C, the samples were deproteinized with 375 µl of sodium perchlorate and incubated at 60^0C for 20 minutes. Subsequently, the samples were filtered through glass fiber filters of pore size 1.2µm. The filters containing microplastics were then dried overnight at 60^0C and examined using a stereomicroscope (Nikon SMZ 1000). Background contamination from the atmosphere was accounted for by using blank samples, and the corresponding values were subtracted from the sample data to ensure accuracy.

3.3.2. Visual assessment and measuring microplastics

Microplastics were categorized in accord to their shape, colour, and size (Gallagher et al., 2016). Based on their shapes, microplastics were classified as fibers, fragments, foam, film, round, and pellet (A. L. Lusher et al., 2017). The surface colour of each identified plastic particle was recorded and categorized as red, black, grey, green, yellow, or white. The size of the microplastics was determined using Nikon Imaging Software (NIS).

3.3.3. Identification of microplastics using Raman spectroscopy

Microplastics were verified using a digital laser Raman spectrophotometer (Renishaw, UK, Model No. M-9836-3991-01-A) installed at the National Centre for Earth Science Studies (NCESS), Thiruvananthapuram, India. The analysis involved a 785nm excitation laser and a grating with 1200 grooves per mm, with 50X objectives and an integration time of 10 seconds. Spectral peaks were evaluated using the Wiley KnowItAll Raman Spectral Library. Matches with a confidence level of 80% or above were accepted.

3.3.4. Statistical analysis

The statistical analysis was conducted using R4.0.2 software. The normality and homogeneity of the data were assessed using the Shapiro-Wilk and Levene's tests,

respectively. To determine if there were significant differences in microplastic abundance among the water samples and bivalve tissue across sites, non-parametric Kruskal-Wallis and Wilcoxon matched pair tests were employed. The Pearson correlation coefficient was applied to investigate the relationship between microplastic burden in surface water and bivalve tissue. Correlation analysis was also performed to evaluate the association between microplastic abundance in bivalves and morphometric parameters including shell length and tissue weight. The criterion for significance was fixed at $P = 0.05$.

3.4. Results and Discussion

3.4.1. Microplastic Quantification

3.4.1.1. Surface water

Microplastic was detected in all the study areas, with the highest abundance (average: 163.67 items L^{-1}) observed in Aluva, Periyar River (S4) and the lowest (average: 31.8 items L^{-1}) in Sathar Island, Vembanad Lake (S5). The microplastic counts varied significantly among the sampling locations ($P < 0.05$), except for Dalavapuram, Ashtamudi Lake (S1) and Kayamkulam Lake (S2), where no significant difference was found ($P > 0.05$). The mean abundance of microplastics in surface water from different sampling sites is presented in Fig 3.1. The study found a high concentration of microplastics in Aluva, Periyar River (S4) near an industrial hub. The continuous discharge of untreated domestic and industrial waste into the lower sections can be attributed to the elevated levels of microplastics in this area (Salas et al., 2017). The current study found larger numbers of microplastics than previous investigations in the Red Hill Lakes in Chennai, India which reported 5.9 microplastics L^{-1} (Gopinath et al., 2020) and the Ganges River in India, which reported 0.05 microplastics L^{-1} (Napper & Thompson, 2016). The increased prevalence of microplastics observed in this study, compared to previous reports can be attributed to the methodology adopted in addition to other factors. Microplastic concentrations were lower in Sathar Island, Vembanad Lake (S5). This island is considered remote and experiences mild impact due to its relatively low population density of 1262 Sqm^{-1} (Wikipedia contributors, 2021), in contrast to the higher population density of the district, which is 2770 Sqm^{-1} (District Administration, 2022)

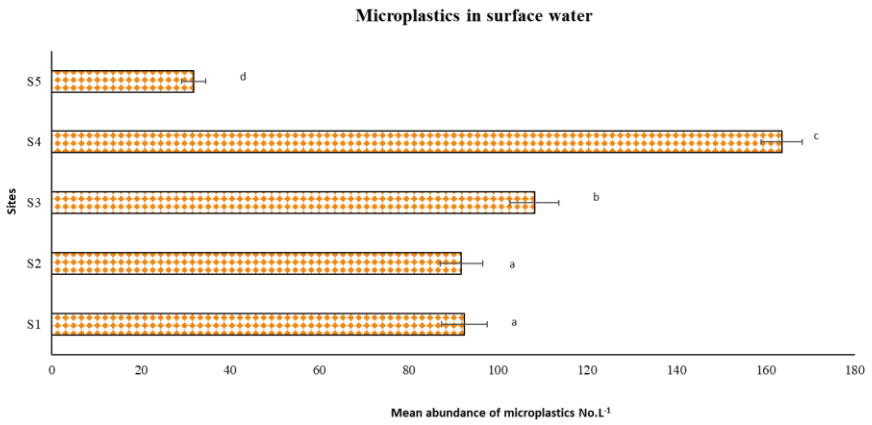

Fig. 3.1. Mean abundance of microplastics in Surface water (±95% confidence interval) for different sites (bars with different letters have significant differences between sites, vertical bars indicate standard error).

3.4.1.2. Bivalve tissue

Microplastics were found in all of the bivalve species studied, with an average of 15.9 items g^{-1} tissue. While the number of microplastics observed in the gills and digestive tissue varied within sampling sites, the difference was not statistically significant ($P > 0.05$). The mean abundance of microplastics in bivalve tissue from different sampling sites is presented in Fig. 3.2. Clams from Aluva, Periyar River (S4) exhibited the highest number of microplastics in both organs (digestive gland mean: 22.8 items g^{-1} tissue and gill mean: 29.6 items g^{-1} tissue). At the same time, mussels from Sathar Island, Vembanad Lake (S5) exhibited the least abundance (digestive gland mean: 5.6 items g^{-1} tissue and gill mean: 8.5 items g^{-1} tissue). In the current study, the abundance of microplastics observed in the gills and digestive glands of three bivalve species was greater compared to previous studies (Narmatha Sathish et al., 2020; (Vasanthi et al., 2021). The elevated concentration of microplastics in the study organisms can be ascribed to ecosystem pollution and the differences in the protocols employed to isolate and quantify the microplastics (A. Lusher et al., 2017). Relative to other techniques, the proteinase-K digestion method exhibited 97% effectiveness when the temperature was regulated at 50^0C for an incubation period of 2h (Cole et al., 2014). Microplastics have also been linked to many detrimental effects in bivalves, such as decreased feeding activity (Besseling et al., 2014), structural changes and inflammatory responses triggered by translocation into the digestive tract and circulatory system (Browne et al., 2008; von Moos et al., 2012). According to a recent study by Frère et al. (2018), microplastics can act as a vector for the oyster pathogen *Vibrio splendidus.*

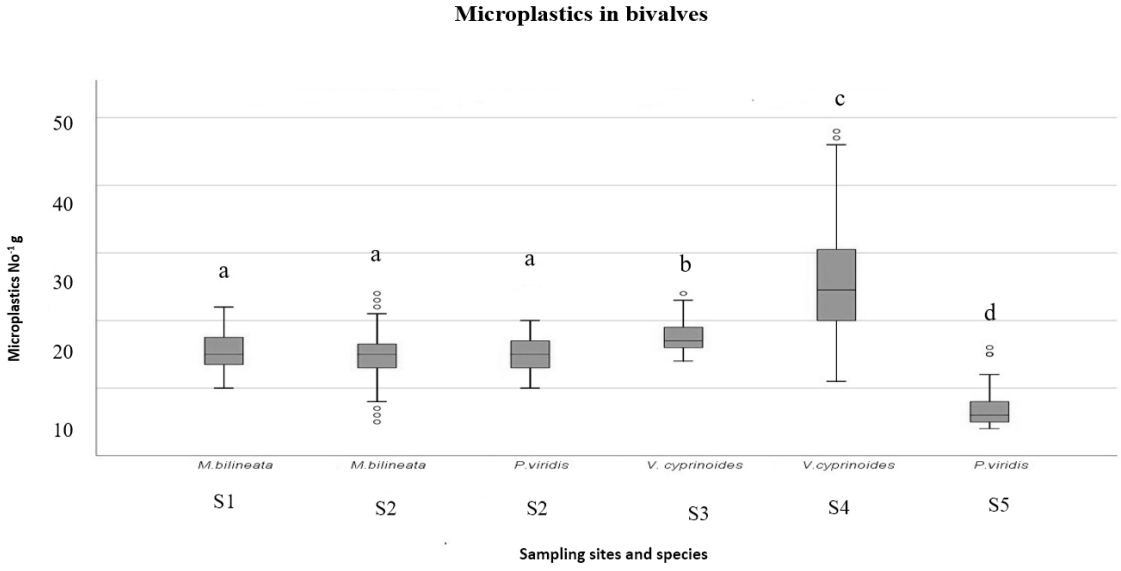

Fig.3.2 Box plot of microplastic abundance in bivalves from different sites (bars with different letters have significant differences between sites).

3.4.2. Correlation of microplastics in bivalves and water

The comparison between the abundance of microplastics in the surrounding water and in bivalve tissues from different sites showed a significant positive correlation ($r = 0.88$, $P < 0.05$) in Pearson correlation coefficient analysis, indicating that bivalve microplastic contamination reflects water microplastic contamination. A study conducted on *P. viridis* and *M. edulis* in coastal waters of China similarly identified a strong positive linear correlation between microplastic concentrations in the water and in the mussels Qu et al., 2018).

3.4.3. Morphology of microplastics in bivalves

Based on their shapes, six different types of microplastics were found in bivalve tissues: round, fiber, fragment, film, foam, and pellets (Fig.3.3). In contrast to other types of microplastics, fibers had distinct colors. Red, black, blue, grey, yellow, green, and white were the commonly observed colours. Across all sites, fibers were found to be the predominant type of microplastics in the gills and digestive tissues of bivalves. This observation is consistent with previous studies that have also identified fibers as the most abundant microplastic type in bivalve tissues (Ding et al., 2021). The textile industries along the banks of Vembanad Lake are likely to be a major source of fiber. The wetlands of Vembanad and Ashtamudi contribute to 70% of the total fish catch in Kerala (Napper & Thompson, 2016). Red (31%) and blue (20%) were the fiber colours observed frequently. This is consistent with reports of commercial bivalves from China (J. Li et al., 2015). Microplastics with sizes ranging from 0.04 to 3.89 mm were found in the present study. The microplastics detected in bivalve tissues were categorized into four size classes (< 1 mm, 1-2 mm, 2-3 mm, and 3-4 mm). Microplastics less than 1mm and between 1 and 2 mm accounted for 32-65% of total microplastics encountered in bivalves across five sampling sites. The size of microplastics plays a crucial role in their bioavailability. Studies in M. edulis and Crassostrea virginica have shown that as the size of plastic particles decreases, their accumulation in the tissues increases (Browne et al., 2008).

3.4.4. Identification of microplastics

Out of 20 microplastic samples examined, polypropylene (PP, 10%) and high-density polyethylene (HDPE, 20%) were the two polymers that were identified by Raman spectroscopy. The spectral peak of detected microplastics is presented in Fig. 3.4. PP and HDPE are considered highly detrimental materials in the aquatic environment due to their persistence. These materials are frequently encountered in the form of fibers and fragments (Blair et al., 2019), originating from various sources such as fishing lines, packaging materials, household plastics, and industrial pipelines (Welden & Cowie, 2017).

3.4.5. Shell length, tissue weight, and microplastic burden in bivalves

There was no clear relationship between the mean microplastic count of bivalves, tissue weight, or shell length. The results were consistent with those of two bivalve species from Oregon (Baechler et al., 2020) and the clam *Atactodea striata* (X.-Y. Xu et al., 2017), where no discernible correlation was found between shell length and microplastic abundance. Studies by Kryger & Riisgard, (1988) suggested that smaller bivalves are more susceptible to

ingesting microplastic than bigger ones because they have a higher relative filtering rate. Moreover, the total microplastic exposure in bivalves may potentially be impacted by species-specific variances.

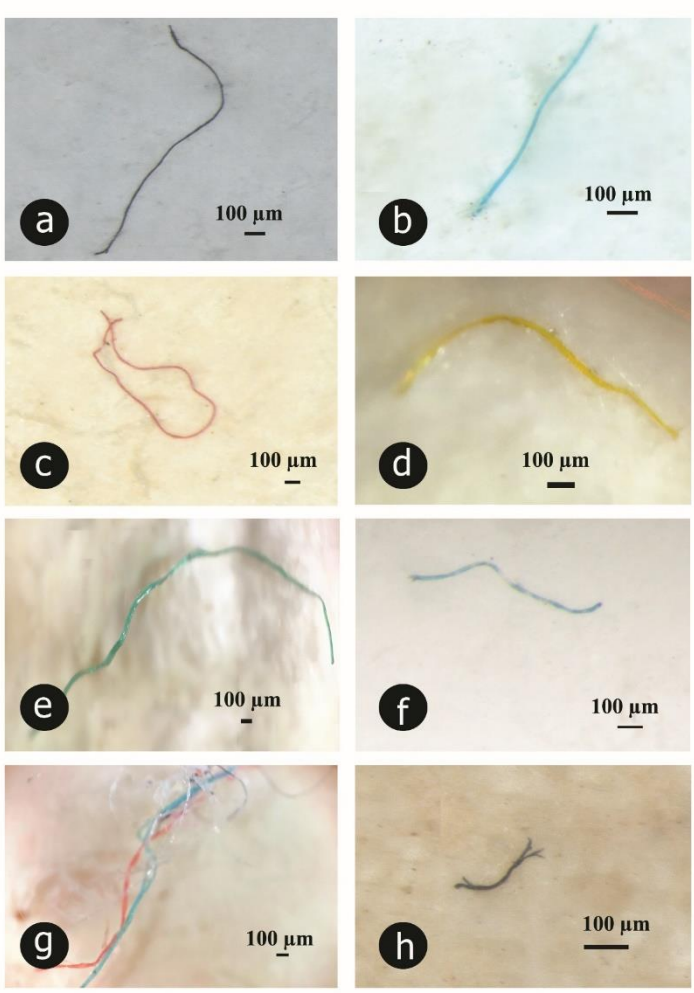

Fig.3.3. Microphotographs of fibers observed in bivalves collected from different sites. a: black; b: blue; c: red; d: yellow; e: green; f: Partially bleached blue; g: multicolor; h: frayed grey.

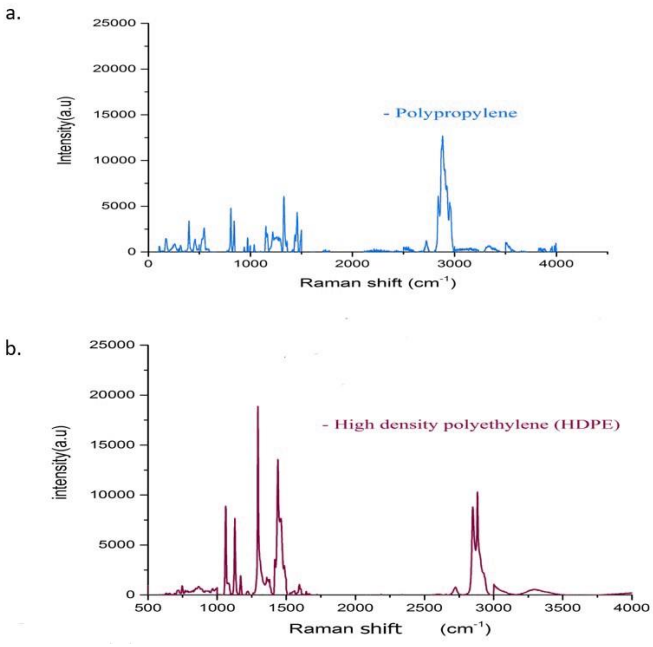

Fig. 3.4. Raman spectra of microplastics in bivalves. a. polypropylene; b. high-density polyethylene.

CHAPTER 4

ASSESSMENT OF CYTOCHEMICAL BIOMARKERS IN BIVALVES

4.1. Summary

Cytotoxicity investigations in bivalves are essential tools for assessing cellular damage and stress response, providing information on the impact of environmental insults on the aquatic ecosystem. Malondialdehyde assay, neutral red retention assay, and acyl CoA assay were used in the present study to analyse the lipid peroxidation, lysosome membrane stability, and peroxisome proliferation, respectively in three bivalve species: edible oyster (*M. bilineata*), green mussel (*P. viridis*), and black clam (*V. cyprinoides*). The highest mean malondialdehyde concentration (4.67 ± 0.9 nmol & 4.72 ± 0.8 nmol) was observed in the digestive gland and gills of *M. bilineata* collected from Dalavapuram, Ashtamudi Lake (S1). The gills of *P. viridis* from Kayamkulam Lake (S2) showed the lowest (1.03 ± 0.8nmol) mean malondialdehyde concentration. The lowest neutral red retention durations were found in *V. cyprinoides* and *M. bilineata* obtained from Aluva, Periyar River (S4) (21.8 ± 1.9), Muhamma, Vembanad Lake (S3) (25.8 ± 2.4), and Dalavapuram, Ashtamudi Lake (S1) (29 ± 1.8), Which were lower than the environmental assessment criteria and indicates that the organisms are considerably stressed. Minimal acyl CoA oxidase activity was measured in the digestive gland of bivalves across sites ranging from 0.6 to 1.00 Mu/mg protein. These cytotoxicity assays are reliable indicators of bivalve health and may also function as sensitive biomarkers for cellular stress induced by aquatic contaminants.

4.2 Introduction

Cellular biomarker responses have the greatest potential for detecting pollution-induced stress in aquatic organisms and are routinely used as a diagnostic tool in biomonitoring programs. Lipid peroxidation is a widely recognized cellular damage response in animals that can be used as an indicator of oxidative stress in cells and tissues (Armstrong & Browne, 1994). Lipid peroxidation occurs when lipids are oxidized, producing primary products like lipid hydroperoxides. Additionally, this process produces various by-products, including bioactive aldehydes. Malondialdehyde (MDA) is the most common type of aldehyde formed. It is a low molecular weight compound that is used as an oxidative stress biomarker (Dargel, 1992). Numerous studies have been performed to evaluate the effectiveness of the MDA assay as a biomarker for assessing oxidative stress in marine invertebrates (Ribera et al., 1991; Breitwieser et al., 2016; Khoei, 2022). Lysosomes are organelles within cells that play vital roles in various essential functions such as nutrition, membrane turnover, cell detoxification, gamete resorption, and cellular defense (Oubella et al., 1994). Many chemical stressors such as heavy metals and organic pollutants have been documented to disrupt lysosomal membranes, with damage proportional to the stress level. Hence, the destabilization of lysosomes has been employed as a valuable indicator of cellular damage in diverse fish and shellfish species (Ringwood et al., 1998; Nicholson, 2001; Zorita et al., 2008). The neutral red retention assay is an in vivo cytochemical technique that relies on the retention of neutral red lipophilic dye. When organisms are exposed to pollutants-

induced stress, the membrane-associated proton pump responsible for lysosomal stability can destabilize. As a result, neutral red leaks out from lysosomes into the cytosol. Conversely, healthy cells can take up and retain larger amounts of the dye. Therefore, the ability of the cells to retain the stain is utilized as an indicator of cellular well-being (Ringwood et al., 1998). Many investigations have found that bivalve hemocytes can accumulate high levels of metals, decreasing lysosome membrane stability (Matozzo, L. Ballarin, D. M. Pampani, 2001). Peroxisomes are organelles surrounded by a single membrane and found throughout eukaryotic cells. They have a crucial role in various metabolic processes, including cellular respiration, which involves the breakdown of H_2O_2 and lipid metabolism (Cancio et al., 1998). Peroxisome proliferation in bivalve molluscs has been studied in both laboratory and field experiments (Cajaraville et al., 2003). Literature on cytotoxicity studies on wild bivalves from the Indian coast is scarce (Niyogi et al., 2001; Jana et al., 2013). Therefore, the current study aims 1) to measure malondialdehyde concentration in the gills and digestive gland tissues of bivalves; 2) to determine the neutral red retention time in the lysosomes in the hemolymph of bivalves; 3) to evaluate the acyl CoA oxidase activity in the gills and digestive gland tissue of bivalves; 4) to understand the spatial variation in the cytotoxicity in bivalves from different sampling sites.

4.3. Materials and Methods

4.3.1. Cytotoxicity

4.3.1.1. Malondialdehyde (MDA) assay

A total of 216 bivalves were examined. The sampling was made during 2019 and 2021. Samples were transported alive in an ice box to the laboratory. The tissues of the gills and digestive glands were dissected out and homogenized on ice using 300 µL of MDA lysis buffer with 3µL butylated hydroxytoluene (BHT). The samples were centrifugated at 13000xg for 10 min to discard any insoluble materials. Subsequently, 200µL of the resulting supernatant was combined with 600µL of thiobarbituric acid (TBA) solution to obtain the MDA-TBA adduct. The mixture was incubated at a temperature of 95^0C for 60 minutes, followed by cooling to room temperature in an ice bath for 10 minutes. MDA reacted with TBA to produce a pink-colored complex, the absorbance was then measured at a wavelength of 532 nm using a spectrophotometer (Multiskan Sky High, Thermo Scientific). The MDA content in unknown samples was determined by comparing their readings to a previously determined MDA standard curve. Results were expressed as nmol/mL. The Sigma-Aldrich (USA) MDA assay kit was used for the sample preparation and biochemical analysis.

4.3.1.2. Neutral red retention (NRR) assay

A total of 180 bivalve samples were examined. The sampling was made from 2021 to 2022. Samples were transported alive in an ice box to the laboratory. The NRR assay was performed according to the protocol by Lowe & Pipe (1994). Hemolymph (0.5 ml) was extracted from adductor muscles and added to siliconized microtubes containing the same amount of physiological saline. Hemolymph (40 µl) was spread on a glass slide and incubated with 40 µl of a neutral red in a light-proof humidity chamber at 18^0C. After 15

minutes, the excess dye was drained and the slides were sealed with a coverslip. Every 15 min, slides were examined under Nikon eclipse 80i and the percentage of cells with dye loss from lysosomes in each field was calculated using the scoring criteria proposed by Martínez-Gómez et al. (2015) (Table 4.1). For each time point, 10 fields with 8-10 cells were randomly observed.

The assay's endpoint was defined as the time when 50% of the cells showed signs of lysosomal leaking. The scoring was terminated at 120 minutes. The total score of the lysosomal condition was calculated as:

$$\%LMS = \left(1 - \left(\frac{\Sigma\, ws}{75}\right)\right) \times\ 100$$

Where $\Sigma\, ws$ is the sum of weighted scores.

Pathology	Score
No effect	0
Enlargement but no leakage	1
Leakage but no enlargement	2
Leakage and enlargement	3
Leakage and enlarged but colorless lysosomes	4
Rounded up fragmenting cells	5

Table 4.1. Scoring criteria for the determination of lysosomal membrane stability.

4.3.1.3. Acyl CoA assay

A total of 216 bivalves were analyzed for acyl CoA assay. The sampling was made during 2019 and 2021. Samples were transported alive in an ice box to the laboratory. The activity of the peroxisomal enzyme acyl-CoA oxidase (AOX) was measured spectrophotometrically (Thermoscientific, Multiskan Sky high) in bivalve digestive gland tissue. After being homogenized in TVBE buffer, samples were centrifuged at 500xg for 15 minutes at 4^0C. Supernatants were transferred to new tubes and diluted with TVBE buffer (1:10). The acyl CoA oxidase (AOX) activity was determined by measuring the quantity of peroxisomal H_2O_2 generated during the oxidation of 30mM palmitoyl CoA. Horseradish peroxidase was used to catalyze the H2O2-dependent oxidation of leuco-dichlorofluorescein to dichlorofluorescein (Small et al., 1985). The reaction medium was prepared, transferred to 2mL aliquots, and kept in the dark at -20^0C until used. 100µL of the sample (1:10) was added to an aliquot with reaction media and incubated for 5 minutes at 25^0C in a water bath. After that, palmitoyl-CoA was added, and dichlorofluorescein production was measured at 502 nm for 4 minutes. Protein concentrations was also determined by the Lowry method (Lowry et al., 1951). AOX activity is expressed as mUnits AOX/mg protein equivalent to nmol H2O2/min/mg protein.

4.3.2. Statistical analysis

The statistical analysis was performed using SPSS 13.0 for Windows. The normality and homogeneity of the data were assessed using the Shapiro-Wilk and Levene's tests, respectively. To determine if there were significant differences in MDA, NRR assay, and acyl co A assays across sites, non-parametric Kruskal-Wallis and Wilcoxon matched pair tests were employed. A significance level of P = 0.05 was used as the threshold for determining statistical significance.

4.4. Results and Discussion

4.4.1 Cytotoxicity

4.4.1.1 Malondialdehyde assay

The digestive glands and gills of *M. bilineata* from Dalavapuram, Ashtamudi Lake (S1) had significantly higher MDA levels (4.67 ± 0.9 nmol & 4.72 ± 0.8 nmol). Meanwhile, the gills of P. viridis obtained from Kayamkulam Lake (S2) had the lowest MDA level (1.03 ± 0.8nmol). The results suggest that the digestive gland and gills are actively involved in producing oxidative stress, making them potential biomarkers for pollution. The digestive glands of bivalves were found to exhibit the highest level of MDA across all sampling sites, except oysters, where slightly elevated MDA concentrations were observed in the gills. The mean malondialdehyde concentrations in the digestive gland and gills of bivalves collected from different sites are presented in Fig. 4.1. A rise in MDA was observed by De Lafontaine et al. (2000) in *D. polymorpha* collected from locations with varying levels of pollution. Heavy metal accumulation studies in bivalves collected from Dalavapuram, Ashtamudi Lake (S1) revealed the presence of toxic heavy metals such as iron, zinc, manganese, copper, cadmium, nickel, lead, and chromium in the digestive gland tissues of the analyzed bivalves, which could be associated with elevated levels of malondialdehyde. Similarly, a study by Gabr et al. (2020) on *Ruditapes decussatus* from the Mediterranean Sea found elevated levels of MDA and heavy metals such as cadmium, copper, lead, manganese, and zinc in the soft tissues. Vlahogianni & Valavanidis, (2007) examined the detrimental impact of copper, cadmium, and lead on *M. galloprovincialis,* which resulted in an elevation in the concentration of MDA. The study conducted by Telahigue et al. 2022 revealed higher levels of MDA in Brachidontes pharaonis from the Tunisian Coast with a higher metal pollution index. A study found that lead had a positive correlation with MDA levels, cell membrane damage, and increased oxidative stress (Chalkiadaki et al., 2015).

4.4.1.2. Neutral red retention assay

The mean neutral red retention time in bivalves collected from different sites is presented in Fig. 4.2. The neutral red retention time ranged from 21.8 to 62.71 minutes. The values were assessed according to the background assessment criteria (BAC) and

environmental assessment criteria (EAC) developed by Martínez-Gómez et al. (2015). Neutral red retention time (NRRT) less than the EAC threshold indicates that the bivalve analyzed is significantly stressed and likely exhibiting pathology. NRRT is less than BAC, but more than EAC and represents a stressed but compensating organism. Higher durations (mean ± SE) were observed in *P. viridis* obtained from Sathar Island, Vembanad Lake(S5) (62.29 ± 5.6), and Kayamkulam Lake (S2) (60 ± 0.45), had longer durations than EAC but shorter than BAC, indicating that mussels sampled are stressed but compensated. Different stages of hemocytes observed during the NRR experiment and associated pathologies are presented in Fig. 4.3. Lower NRRT was observed in *V. cyprinoides* from Muhamma, Vembanad Lake (S3) (25.8 ± 2.4), and Aluva, Periyar River (S4) (21.8 ± 1.9), as well as in *M. bilineata* from Dalavapuram, Ashtamudi Lake (S1) (29 ± 1.8). In all these cases, NRRT levels were below the EAC threshold, indicating organisms were severely stressed, unhealthy, and had completely destabilized lysosomes, indicating pollution. According to the assessment standards, none of the bivalves examined in this study were considered healthy (NRRT ≤ 120). The highest mean lysosomal stability (69.7 ± 12 %) was in *P. viridis* collected from Sathar Island, Vembanad Lake (S5). The lowest LMS observed in bivalves collected from Aluva, Periyar River (S4) (45 ± 10 %) can be attributed to the discharge of effluents from chemical industries situated along the river banks (Salas et al., 2017). The study conducted by Yancheva et al. (2016) revealed that the lysosomal membrane in zebra mussels is destabilized due to exposure to lead and nickel. This finding aligns with the observation of lower NRRT and increased levels of nickel and lead in the digestive gland tissues of bivalves obtained from Muhamma, Vembanad Lake (S3), and Dalavapuram, Ashtamudi Lake (S1). Additionally, the NRR assay conducted on clam hemocytes *(Mya arenaria)* also demonstrated low NRRTs (0-15 minutes) at a contaminated site along the Romanian Black Sea coast (Ciocan, 2002). The decreased LMS observed in this study suggested stress and damage in the hemocytes of bivalves due to exposure to environmental contaminants.

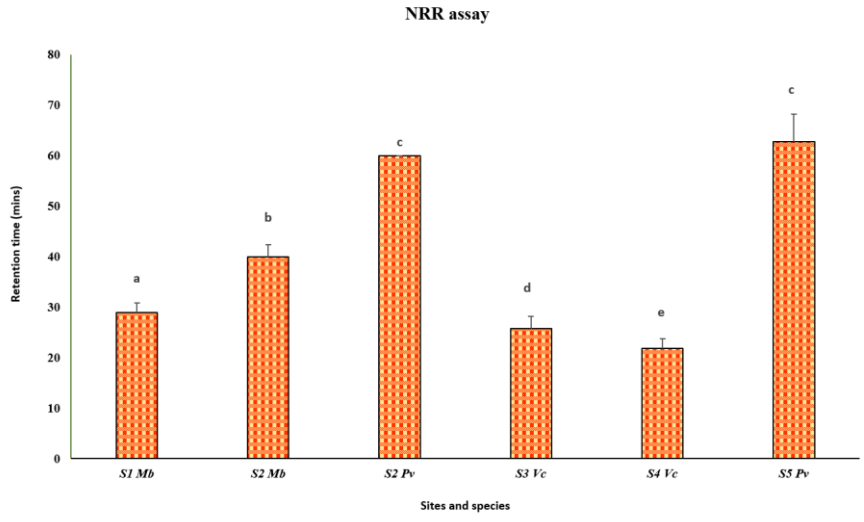

Fig.4.2. NRRT (Mean ± SE) in the lysosome of bivalves (bars with different letters have significant differences between sites, vertical bars indicate standard error). Mb: *M. bilineata*, Pv: *P. viridis*, Vc: *V. cyprinoides.*

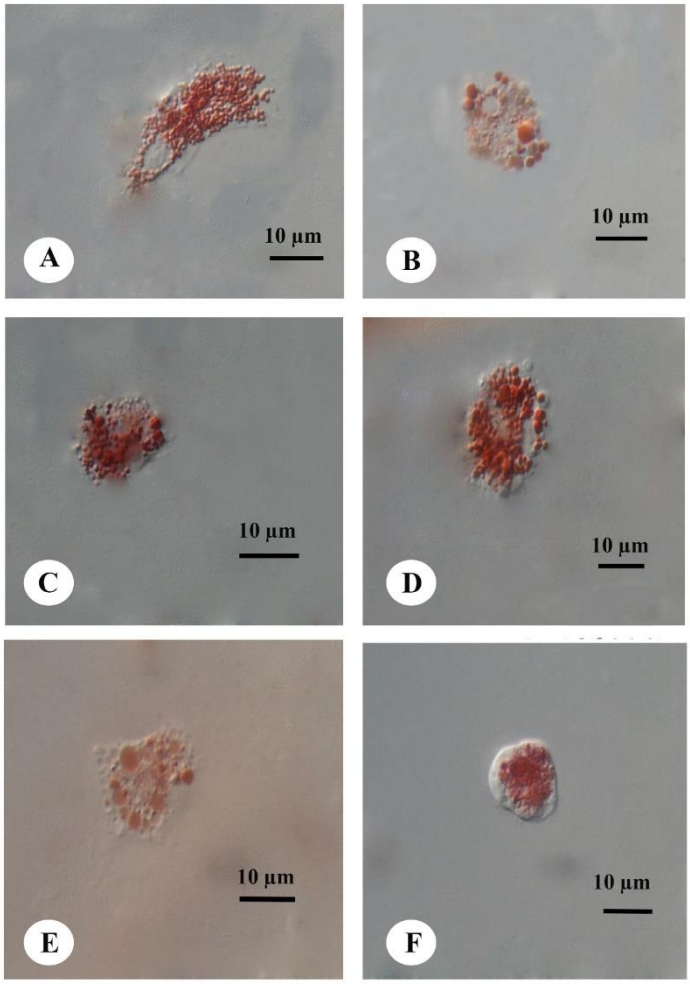

Fig.4.3. Microphotographs of bivalve hemocytes showing uptake of neutral red in lysosomes: A: no effect; B: enlargement but no leakage; C: leakage but no enlargement; D: leakage and enlargement; E: leakage and enlarged but colorless lysosomes; F: rounded up fragmenting cells.

4.4.1.3. Acyl CoA assay

The mean peroxisomal acyl-CoA oxidase activity in the digestive gland of bivalves across different sites is presented in Fig. 4.6.

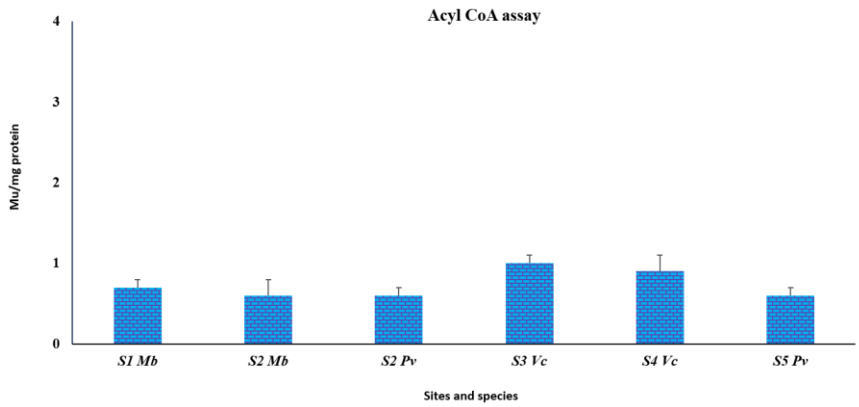

Fig.4.4. Acyl CoA assay (mean ± SD) in the digestive gland of bivalves (vertical bars indicate standard deviation). Mb: *M. bilineata*, Pv: *P. viridis*, Vc: *V. cyprinoides*.

There were no statistically significant differences in AOX activity across sites. Almost all bivalve digestive glands examined had minimal peroxisomal AOX activity ranging from 0.7 to 1.00 Mu/mg protein. Numerous studies have extensively demonstrated that trace metals have a significant impact on the metabolism of organic xenobiotics, operating through a complex network of interactions. In a prior experiment when mussels were exposed to benzopyrene and cadmium either alone or together, cadmium suppressed the reaction that benzopyrene induced (Orbea et al., 2002). The inhibitory effects of trace metals on peroxisomal AOX activity have been proposed as the underlying cause for the restricted variability observed in this enzyme (Bocchetti et al., 2008). Several studies have also indicated that copper does not induce peroxisome proliferation in mussels. These observations suggest that the peroxisome proliferation response is specific to organic xenobiotics rather than metals (Cajaraville & Ortiz-Zarragoitia, 2006).

CHAPTER 5

ASSESSMENT OF GENOTOXICOLOGICAL BIOMARKERS IN BIVALVES

5.1. Summary

Genotoxicity biomarkers are envisaged as key molecular indicators of toxic effects from environmental contaminants in ecotoxicology studies. Comet and micronucleus assays were used to detect DNA damage and chromosomal abnormalities in the hemocytes and gill cells of edible oyster *(M. bilineata)*, green mussel *(P. viridis)*, and black clam *(V. cyprinoides)* from internationally significant ecosystems along the south-west coast of India. The highest abundance of micronuclei (9.47 MN/ 1000 cells) and DNA damage (12.59 ± 5.3) was observed in *V. cyprinoides* from Muhamma, Vembanad Lake (S3). Nuclear abnormalities such as nuclear buds, nucleoplasmic bridges, and binucleated cells were also observed across sites, while *M. bilineata* and *P. viridis* from Kayamkulam Lake (S2) had the lowest micronuclei incidence and DNA damage. Significant differences in MN and DNA damage were found across sites and the study indicated that the sampling sites were low to moderately polluted with genotoxic substances.

5.2. Introduction

The assessment of genotoxicity biomarkers, which serve as a molecular indicators of the harmful impacts of environmental pollutants, is extensively employed in the discipline of ecotoxicology. The two most frequently applied tests for detecting DNA damage and chromosomal abnormalities in aquatic species are the comet and MN assay (Bolognesi & Cirillo, 2014). Acentric chromosome fragments or entire chromosomes that lag during the metaphase or anaphase transition as a result of clastogens or aneugens generate MN, which is evident as a tiny secondary nucleus in interphase (Mersch & Beauvais, 1997). Genotoxicity studies in bivalves also demonstrated the presence of nuclear aberrations like NB, NPB, and BN cells together with MN as valuable indicators for assessing the genotoxic effects of

contaminants (Butrimavičienė et al., 2018). Several reports have confirmed the presence of MN in crabs, fishes, worms, and bivalves exposed to heavy metals, PCBs, and PAHs under field and laboratory conditions (Aborgiba et al., 2016; Braham et al., 2017). The comet assay or single-cell gel electrophoresis is a versatile method for assessing DNA damage, including single and double-strand breaks induced by genotoxic contaminants (Praveen Kumar et al., 2014). Comet assay has so far been used successfully on a wide range of taxa including polychaetes, crustaceans, bivalves, gastropods, and fishes (Lewis & Galloway, 2008; Parrella et al., 2015; Vernon & Jha, 2019; M. Karim, 2022; Pandey et al., 2018). A few recent field studies have been published that assess MN and DNA damage in bivalves, with a particular emphasis on various pollutants in the aquatic environment (D'costa et al., 2018; Salem Al-Howiti et al., 2020). The gills and hemolymph of bivalves serve as valuable organs for assessing the effects of genotoxic pollutants. Genotoxicity studies in Indian bivalves, particularly in its diverse and ecologically significant regions, are notably scarce (Revankar & Shyama, 2009; Dias et al., 2021). Hence the present study aims 1) to measure the frequency of MN, NPB, NB, and BN in the gill cells of bivalves; 2) to understand the seasonal and geographical variance in MN across different sampling sites; 3) to assess the DNA damage in hemocytes of bivalves sampled from different sites.

5.3. Materials and Methods

5.3.1 Genotoxicity

5.3.1.1. Micronucleus assay

A total of 360 samples were analyzed. Samplings were made during pre-monsoon, monsoon, and post-monsoon of the year 2019 and 2021. A section of the gill tissue was carefully dissected from each sampled organism and placed on a glass slide with a drop of methanol-acetic acid solution (3:1). The gills were gently nipped with needles for a few minutes to create a cell mixture, which was then spread and allowed to air dry. The smears were fixed in methanol for 10 minutes and stained with 5% Giemsa solution prepared in phosphate buffer (pH 6.8) for 15 minutes (Barsiene et al., 2004). Three slides were made for each bivalve, and samples were coded and analyzed blindly. Following the criteria outlined by Fenech et al. (2003), the frequency of nuclear abnormalities (MN, NPB, NB, and BN) was calculated by examining 1000 intact cells per slide at 1000x magnification. Images were captured using a Nikon Eclipse 80i microscope.

5.3.1.2. Comet assay

A total of 90 samples were analyzed. Samplings were made during the post-monsoon of 2021 and pre-monsoon and monsoon of 2022. Hemolymph (300µl) was collected with a syringe from the adductor muscles of each sampled bivalve and transferred to an ice-cold microcentrifuge tube. The cell viability and counts were examined before the assay to confirm there were sufficient live cells to conduct the comet assay (JanakiDevi et al., 2013). The comet assay was performed according to Praveen Kumar et al. (2014). A volume of 15µl of hemolymph was mixed with 75 µl of 0.5 % low melting agarose at 37^0C and evenly spread over a microscopic slide previously coated with 1% normal melting point agarose. After

covering the slides with a coverslip, they were cooled in a refrigerator at a temperature of 0^0C for 5 minutes to allow the agarose to solidify. Subsequently, the coverslip was cautiously removed, and the slide was immersed in a newly prepared cold lysing solution inside a sealed container. The slide remained in the lysing solution for 2 hours at a temperature of 4^0C. The DNA was then allowed to unwind for 30 minutes by placing comet slides in an electrophoresis buffer and electrophoresed for 20 minutes at 20 V and 275mA. The unwound DNA was neutralized in a cold neutralization buffer for 10 minutes, washed in distilled water, and neutralized again. All the preceding steps were carried out in a dark environment to prevent photooxidation of DNA. The DNA was then stained with 15 µg/ml ethidium bromide and observed under a Nikon Eclipse 80i fluorescence microscope at 1000x magnification. Photomicrographs were taken using NIS elements imaging software. The images were analyzed employing Comet Assay Software Project (CASP) image analysis software (Końca et al., 2003), and the content of tail DNA was enumerated. Each bivalve was examined in duplicate slides, and 25 cells were counted on each slide.

5.3.2. Statistical analysis

The data were statistically analyzed using SPSS 13.0 for Windows. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to determine the normality and homogeneity of the results. Non-parametric tests like Kruskal Wallis and Wilcoxon matched pair tests were used to examine significant differences in nuclear abnormalities and DNA damage between zones and seasons. The P value for the significant threshold was set at 0.05.

5.4. Results and Discussion

5.4.1 Genotoxicity

5.4.1.1 Micronucleus assay

Bivalve gill cells revealed a variation in the frequency of MN, NB, BN, and NPB cells across each site. The descriptive statistics of nuclear abnormalities in bivalve gill cells from distinct sampling sites are presented in Fig. 5.1. Higher frequencies of MN (mean ± SD) /1000 cells were observed in the *V. cyprinoides* from Muhamma, Vembanad Lake (S3) (9.47 ± 1.80) compared to bivalves from other sites. *Magallana bilineata* and *P. viridis* from Kayamkulam Lake (S2) had the lowest values of (4.38 ± 1.3) and (4.55 ± 1.1) MN / 1000 cells, respectively. Significant differences in MN were found across five sites ($P < 0.05$). Studies have shown that xenobiotics in the aquatic environment can elevate the frequency of micronuclei in bivalves (Badino et al., 1991). Therefore, the MN observed in the current study can attributed to the presence of xenobiotics in the aquatic ecosystem. Micronuclei are tiny chromatin fragments that are detached from the main cell nucleus and are indicative of chromosomal breakage or mitotic spindle malfunction (Krupina et al., 2021). They are formed in all types of cells following irregular cell division processes in which a chromosome fragment or entire chromosome is not eliminated during anaphase but is delayed relative to the rest of the chromosomes, forming a small secondary nucleus in the interphase (Baršienė et al., 2008). No significant seasonal variations in MN frequency were observed across sampling sites during 2021 ($P > 0.05$). However, significant ($P < 0.05$) seasonal variations in

the MN frequencies of bivalves sampled from Kayamkulam Lake (S2), Aluva, Periyar River (S4), and Sathar Island, Vembanad Lake during 2019, with the exclusion of *P. viridis* from Kayamkulam Lake (S2) remained constant throughout the seasons. The majority of bivalves analyzed from various locations exhibited a consistent MN frequency with no seasonal variation. This could be attributed to the fact that micronuclei caused by clastogenic and aneugenic agents tend to persist long after exposure to these chemicals (Majone et al., 1990; Majone et al., 1987). Toxicity studies on *V. cyprinoides* collected from Muhamma, Vembanad Lake (S3) in 2019 revealed elevated levels of harmful heavy metals, including Fe, Zn, Mn, Cu, Cd, Ni, Pb, and Cr, in the tissues. This finding is likely associated with the higher frequency of micronuclei observed in bivalves from the same location. However, Contaminants in aquatic ecosystems often occur in complex mixtures, which can lead to interactions between substances and result in unpredictable genotoxic responses to pollution (Baršienė et al., 2015). The frequency of MN observed in *V. cyprinoides* from Muhamma, Vembanad Lake (S3) is similarly high to that reported in previous investigations. Musrri et al. (2021) reported MN rates of 14.4 MN per 1000 cells in hemocytes of the mussel *Perumytilus purpuratus*, sampled from the Chilean coast. BN cell frequency ranged from (2.26 ± 1.6) to (5.46 ± 1.4) per 1000 cells, which was lower than MN. Binucleation is a sign of irregular cell division because it happens when cytokinesis is blocked, which causes a genetic imbalance in the cells and perhaps contributes to carcinogenesis (Rodilla, 1993). In the present study, the mean NB ranged from (2.17 ± 1.3) NB/1000 cells in *M. bilineata* from Kayamkulam Lake to (4.78 ± 0.6) NB/1000 cells in *V. cyprinoides* from Muhamma, Vembanad Lake (S3). NBs, also known as incomplete MN, are abnormalities that resemble MNs in shape, structure, and size and are connected to the main nuclei of cells via chromatin threads or stalks (Fernández et al., 2011).

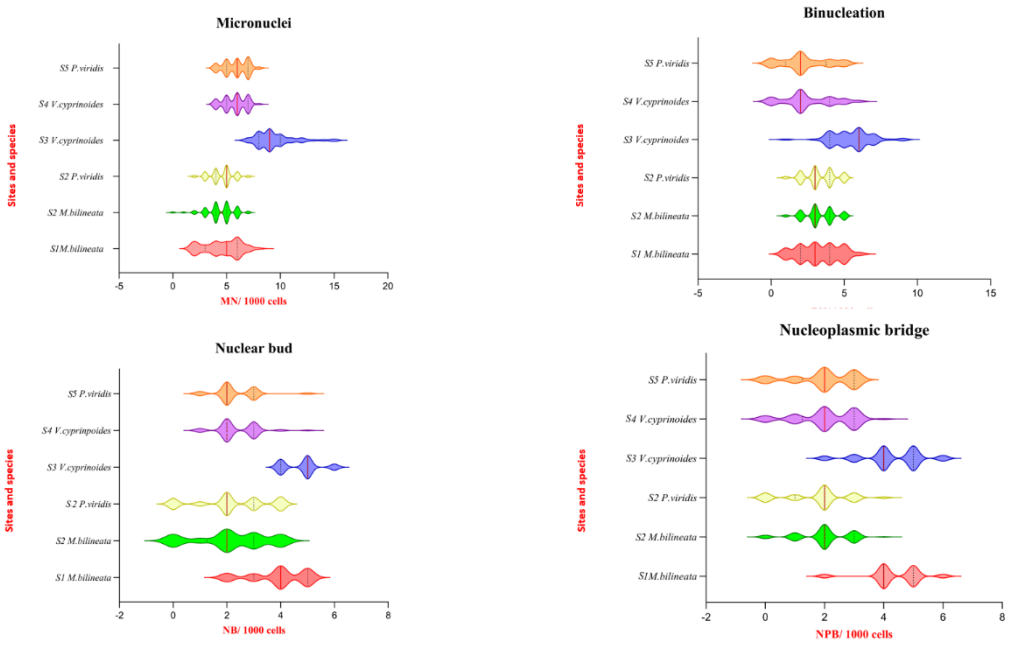

Fig.5.1. Descriptive statistics of nuclear abnormalities in bivalve gill cell.

Analysis of NPB cells showed that their frequency ranged from (1.76 ± 1.05) to (4.4 ± 0.87) per 1000 cells and was typically less frequent than all other abnormalities. Nucleoplasmic bridges are the result of a chromosomal mutation in which the nuclear envelope fails to completely separate the chromosomes of the two daughter nuclei, resulting in the formation of a cytoplasmic bridge (Guidi et al., 2021). . Microphotograph of evaluated nuclear anomalies is presented in Fig.5.2.

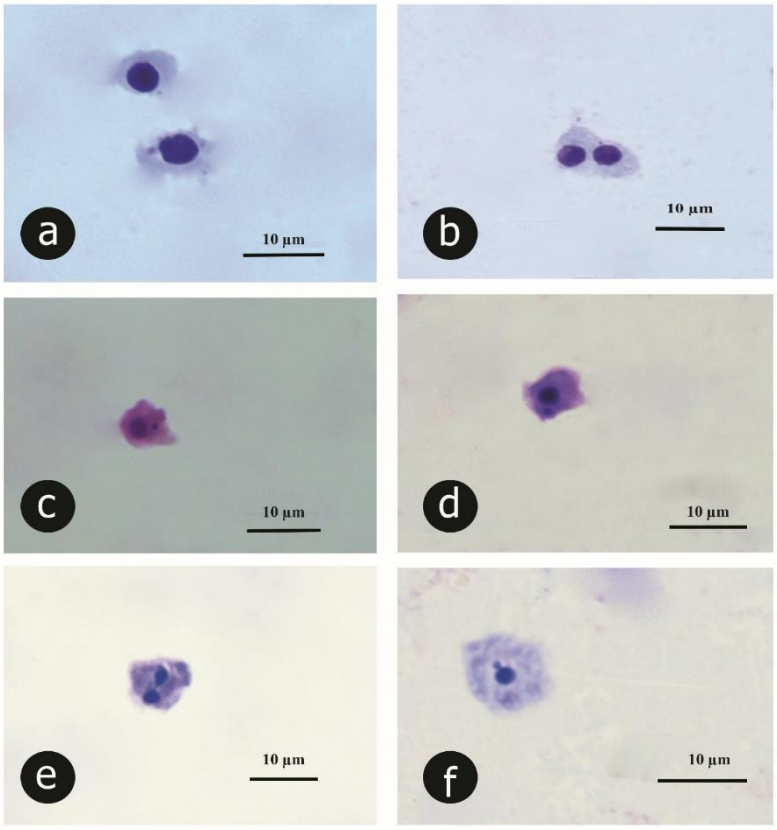

Fig.5.2. Microphotographs of nuclear abnormalities in bivalve gill cells: a: normal cell; b: binucleated cell; c-d: micronuclei; e: nucleoplasmic bridges; f: nuclear bud.

The current study revealed a higher occurrence of nuclear abnormalities in Muhamma, Vembanad Lake (S3). Several authors have reported an increase in the frequency of nuclear abnormalities in bivalves exposed to different pollutants (Sreejamole & Remya, 2014; Baršienė et al., 2015).

5.4.1.2. Comet assay

A significant difference in DNA damage was found in the hemocytes of bivalves sampled from different sites ($P < 0.05$). The findings of breakage of DNA strands, expressed as the mean percentage tail DNA content presented in Fig. 5.3. The percentage of DNA in the comet tail was higher in *V. cyprinoides* from Muhamma, Vembanad Lake (S3) (12.59 ± 0.73), followed by *V. cyprinoides* from Aluva, Periyar River (S4) (10.35 ± 2.3) and *M. bilineata* from Dalavapuram, Ashtamudi Lake (S1) (7.3 ± 1.8). This suggests the presence of DNA-damaging toxicants, most likely trace metals in the aquatic environment, which results in the loss of DNA integrity via DNA strand breaks. In contrast, the lowest percentage of tail

DNA content was observed in *M. bilineata* (2.2 ± 1.0) and *P. viridis* (1.96 ± 1.0) from Kayamkulam Lake.

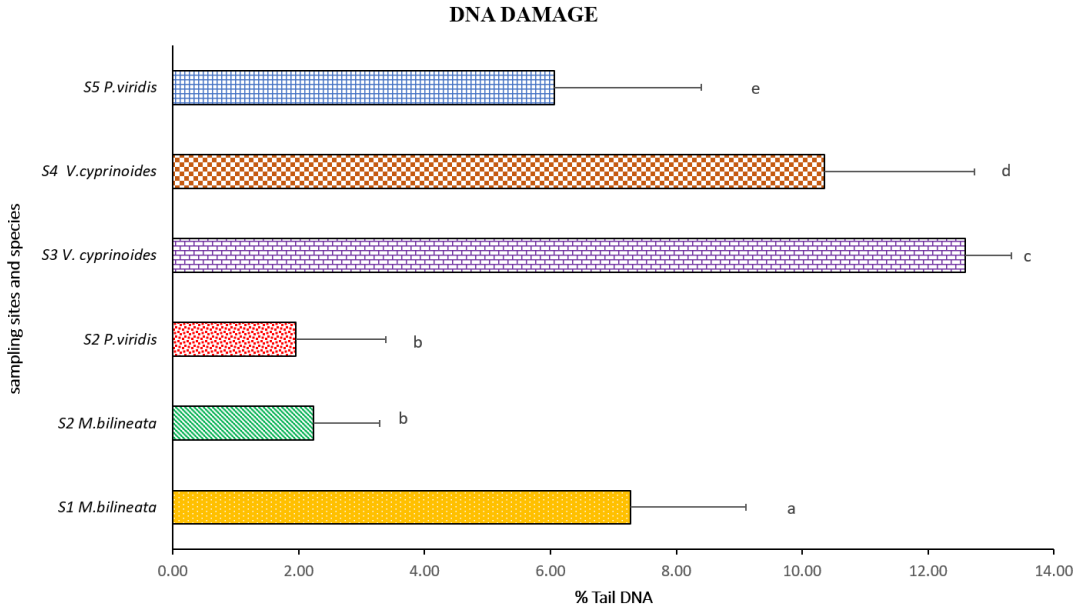

Fig. 5.3. Tail DNA (%) in the hemocytes of bivalves (Mean ± S.D) collected from different sites. (different letters indicate significant differences between zones).

Bivalve hemocyte nuclei with and without DNA damage are presented in Fig. 5.4. The Muhamma, Vembanad Lake (S3) revealed relatively higher levels of heavy metals such as Mn, Ni, Cu, and Cr in the soft tissue of *V. cyprinoides* during previous years. The genotoxic effects of these heavy metals are associated with the accumulation of DNA-damaging free radicals and clastogenic processes or a combination of both clastogenic and aneugenic actions in aquatic organisms (Baršienė et al., 2012). This accumulation may contribute to the increased DNA damage observed in the bivalves. Because chromium-induced laboratory tests on M. edulis revealed DNA damage in the gill tissues in earlier studies (Emmanouil et al., 2007). Furthermore, Rank et al. (2005) discovered a strong relationship between chromium content and DNA damage in *M. edulis* field samples collected from the Danish coast. *P. viridis* from Kayamkulam Lake (S2) had the least DNA damage.

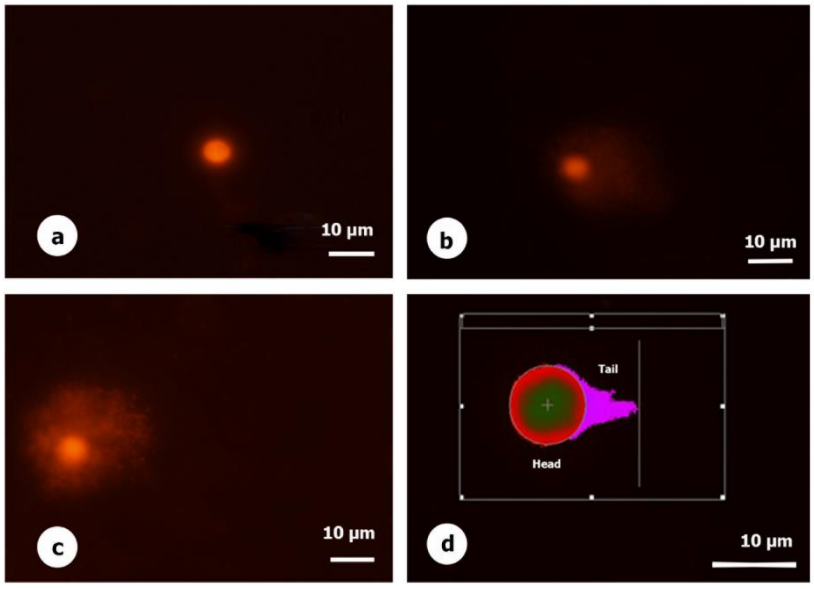

Fig.5.4. Microphotographs of comet images showing different levels of DNA damages: a: undamaged DNA; b-d: damaged DNA.

Except for Cu and Cd, which were present in higher concentrations, all metals examined in bivalve tissues were found at significantly lower concentrations in this site. According to a recent study on cadmium toxicities in marine bivalves, clams were more sensitive to cadmium than oysters, mussels, and scallops (Zhan et al., 2023).

CHAPTER 6

CONCLUSION

In this comprehensive study, several cell, tissue, and genomic level biomarkers were used for the first time on wild populations of *M. bilineata*, *P. viridis* and, *V. cyprinoides* to assess the health status of the ecologically sensitive regions of international significance along the southwest coast of India. The analyzed bivalves revealed histopathological alterations (vacuolation, tubular atrophy, lamellar disorganization, and the presence of prokaryotic inclusions and parasites.), heavy metal toxicity, microplastic contamination, oxidative stress, lysosomal destabilization, peroxisome proliferation, chromosome anomalies and DNA damage across sites. This multi-biomarker approach provides a better understanding of organism health and validates the effectiveness of sedentary bivalves for aquatic biomonitoring programmes. Based on the battery of biomarkers analyzed, the most stressed organism was *V. cyprinoides* from Muhamma in Vembanad Lake (S3), followed by *M. bilineata* from Dalavapuram in Ashtamudi Lake (S1) and *V. cyprinoides* from Aluva in the Periyar River (S4), all of which exhibited moderate to severe abnormalities. it is imperative to implement stringent regulatory measures to prevent further degradation of aquatic health in the future. The bivalve resources in these zones are vital to the fisheries providing an income for many people involved in fishing-related activities. Additionally, these species are highly favoured as a culinary choice by both locals and tourists raising alarming concerns regarding the potential transfer of contaminants into the food web.

PUBLISHED ARTICLES: 2

Joshy, A., Sharma, S.R.K., Mini, K.G., Gangadharan, S., Pranav, P., 2022. Histopathological evaluation of bivalves from the southwest coast of India as an indicator of environmental quality. Aquat. Toxicol. 243, 106076. https://doi.org/10.1016/j.aquatox.2022.106076

Joshy, A., Krupesha Sharma, S.R., Mini, K.G., 2022. Microplastic contamination in commercially important bivalves from the southwest coast of India. Environ. Pollut. 305, 119250. https://doi.org/10.1016/j.envpol.2022.119250

PAPERS PRESENTED: 2

Joshy, A., Gangadharan, S., Shamal, P., Sanil, N.K., Sharma, S.R.K., 2020. Development of histopathological indicators in bivalve clams along the Kerala coast for assessing environmental quality. In: Joshi, K.K., Varghese, M., Kaladharan, P., Sreenath, K.R., Pillai, S.L., Sanil, N.K., Mohamed Hatha, A.A., Shinoj, P., Padua, S., Gills, R. and Jayakumar, C.V. Marine Ecosystem Challenges & Opportunities (MECOS 3), International Symposium, Kochi, pp. 469-470.

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